

# Lab on a Chip

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ARTICLE TYPE

## Pumpless microfluidic platform for drug testing on human skin equivalents

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Advances in bio-mimetic *in vitro* human skin models increase the efficiency of drug screening studies. In this study, we designed and developed a microfluidic platform that allows for long-term maintenance of full thickness human skin equivalents (HSE) which are comprised of both the epidermal and dermal compartments. The design is based on the physiologically relevant blood residence times in human skin tissue and allows for the establishment of an air-epidermal interface which is crucial for maturation and terminal differentiation of HSEs. The small scale of the design reduces the amount of culture medium and the number of cells required by 36 fold compared to conventional transwell cultures. Our HSE-on-a-chip platform has the capability to recirculate the medium at desired flow rates without the need for pump or external tube connections. We demonstrate that the platform can be used to maintain HSEs for three weeks with proliferating keratinocytes similar to conventional HSE cultures. Immunohistochemistry analyses show that the differentiation and localization of keratinocytes was successfully achieved, establishing all sub-layers of the epidermis after one week. Basal keratinocytes located at the epidermal-dermal interface remain in a proliferative state for three weeks. We use a transdermal transport model to show that the skin barrier function is maintained for three weeks. We also validate the capability of the HSE-on-a-chip platform to be used for drug testing purposes by examining the toxic effects of doxorubicin on skin cells and structure. Overall, the HSE-on-a-chip is a user-friendly and cost-effective *in vitro* platform for drug testing of candidate molecules for skin disorders.

### Introduction

Development of *in vitro* human skin models has gained significant attention in the past three decades, with the goal of predicting skin-related complications as well as assessing the efficacy of transdermal delivery of new drugs. Human skin equivalents (HSEs) are freestanding constructs comprised of biomimetic compositions of extracellular matrix proteins/lipids and primary human skin cells, such as fibroblasts and keratinocytes. Depending on the application, HSEs can be designed and used as dermal only (fibroblast in collagen matrix), epidermal only (keratinocytes cultured on collagen base), or full thickness (epidermal+dermal) settings.<sup>1</sup> The full thickness model has the potential to outperform the current animal skin or human cadaver models, especially after recent progress in incorporation of induced pluripotent stem cell derived skin cells and primary vascular cells into HSEs.<sup>2</sup>

Recent interest has focused on transferring various microphysiological organ models, including skin, onto

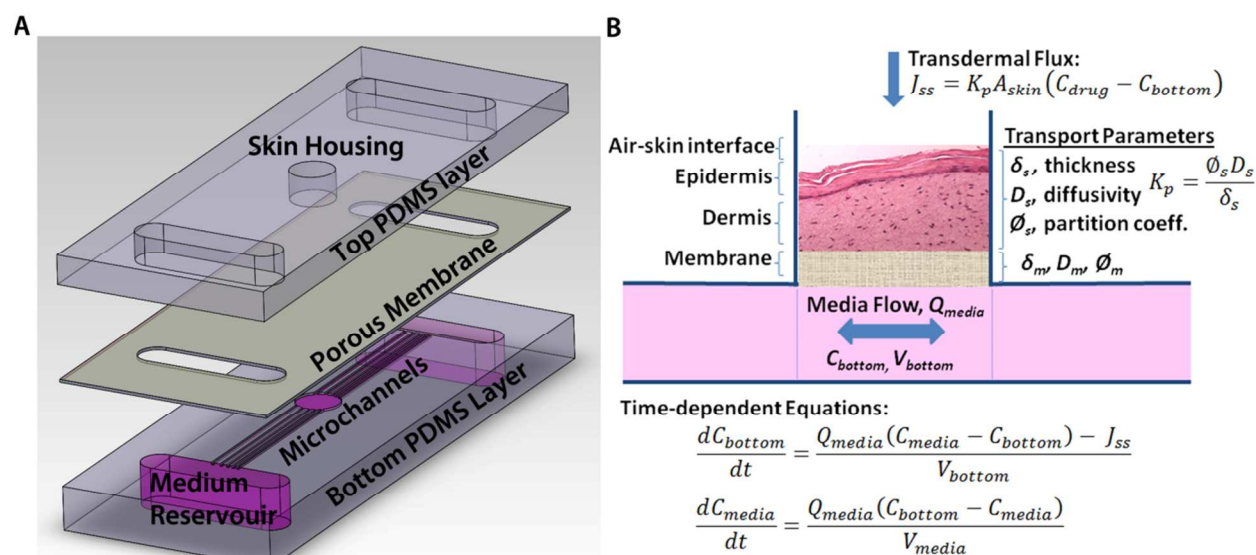
microfluidic platforms to enable better control over physical and chemical factors in cell microenvironment.<sup>3-4</sup> Transfer of *in vitro* skin models into microfluidic platforms enables physiologically relevant transport of nutrients and exogenous substances to the skin tissue, and permits more reliable evaluation of drug candidates in terms of toxicity, efficacy and delivery. Wagner et al<sup>5</sup> and Atac et al<sup>6</sup> used a multi-organ platform, where skin biopsies or a commercial full-thickness skin model (EpidermFT, Mattek, MA) can be co-cultured with human hair or liver microtissues. Media perfusion in both of these studies was dependent on the use of an integrated micropump. Successful long-term maintenance of HSEs in these platforms highlights the potential utility of skin-on-chip systems in drug testing studies.

In this study, we designed and developed a pumpless HSE-on-a-chip platform which is simple to fabricate, handle and operate. Recirculating gravity-driven flow was achieved by placing the HSE-on-a-chip device on a rocking platform, as demonstrated previously for other organ-on-chip systems.<sup>7-8</sup> The HSE-on-a-chip platform was designed to have a physiological residence time of blood in the tissue so that relevant concentration profiles

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**Fig.1 Description of the microfluidic system and transport model (A)** Scheme of the HSE-on-a-chip platform with each individual component **(B)** Description of the transport model geometry and parameters.

of drugs in blood can be achieved after topical delivery. This feature also allows for future integration of the system into multiple organ settings. We validated the use of this system for drug testing purposes by examining known skin-related toxicity of the anti-cancer drug, doxorubicin. Overall, we demonstrate for the first time that full-thickness HSEs can be maintained long-term and used for drug testing in a micro-scale setting without the need for pumps or external tubing.

## Materials and Methods

### Cell Culture and Preparation of Full-thickness Human Skin Equivalents

Fibroblasts and keratinocytes were derived from human foreskin, with fibroblasts cultured in DMEM with 10% FBS and keratinocytes in EpiLife (Life Technologies). 3D skin equivalents were generated similar to the method described previously (all media formulations can be found in refs [9 and 10]). Briefly, 4 ml of type I collagen matrix containing  $1.25 \times 10^5$  fibroblasts/ml was deposited onto polyethylene terephthalate membranes (BD Biosciences). After polymerized matrix was cultured for 3-7 days,  $1 \times 10^6$  keratinocytes were plated on the matrix and cultured submerged with epidermalization medium<sup>9</sup> for additional 7 days. Subsequently, cornification medium<sup>9</sup> was applied at a reduced level to allow for culturing the constructs at the air-liquid interface for 7 days before harvesting for analysis or culturing in maintenance medium<sup>9</sup> for drug treatment.

### Microfabrication of the HSE-on-a-Chip Platform

The microfluidic platform is consist of two polydimethylsiloxane

(PDMS) layers separated by a polycarbonate porous membrane with 5  $\mu\text{m}$  pore size (Cyclopore Whatman, GE Healthcare, PA). **(Figure 1A)** The top PDMS layer is 2 mm thick and has a circular housing with a diameter of 6 mm at the center to accommodate the HSEs. The bottom PDMS layer is 3 mm thick and has 5 parallel microchannels of 150  $\mu\text{m}$  in height and width. At both ends of the microchannels there are two media reservoirs that have a total volume of 250  $\mu\text{l}$ . The bottom PDMS layer was attached onto a glass slide after treating both PDMS and glass surfaces with plasma at 18 W for 30 sec. The microchannels were fabricated using standard lithography techniques. Briefly, SU-2100 (Microchem, MA) photoresist was spun at 500 rpm for 10 sec followed by 1800 rpm for 30 sec. The wafer was then soft-baked at 65  $^\circ\text{C}$  and 95  $^\circ\text{C}$  for 5 min and 30 min, respectively. The surface was then UV-exposed for 20 sec, post-baked for 12 min and developed for 15 min. The reservoirs on all layers and the HSE housing on top layer were made by punching through holes at desired dimensions.

The polycarbonate membrane and PDMS layers were bonded at room temperature as described previously.<sup>11</sup> Briefly, the membranes were first rinsed with ethanol and isopropanol, air dried and plasma cleaned for 1 min at 18 W. They were then soaked in 1% v/v (3-Aminopropyl)triethoxysilane (APTES) solution in de-ionized water for 20 min and rinsed with de-ionized water. The PDMS layers treated with plasma at 18 W for 30 sec and then immediately put in contact with the membranes. Irreversible bonding was formed in less than a minute at room temperature.

### Maintenance of HSE-on-a-Chip

The device and all parts were sterilized by autoclaving. The culture medium was introduced into the system by loading the medium in one of the reservoirs and applying negative pressure on the opposite side. Prior to the transfer of skin constructs onto the chip, they were maintained in cornification medium in transwell inserts for 1 week. A circular piece with a radius of 2.5 mm was cut out of the HSEs using a hole-puncher and immediately placed into the housing on the chip. The bottom surface of the skin construct was in close contact with the membrane and medium whereas the top layer was kept in contact with air. The device was put in a petri dish and placed on a rocking platform in a humidified incubator at 37 °C and 5% O<sub>2</sub>. The angle and speed of the rocking platform (VWR, PA) was 15 degrees and 2 cycles/min, respectively, allowing for the desired flow rates and residence times. The medium was replaced every other day. The HSEs from different time points were collected from separate chips running in parallel to prevent any interference in tissue culture conditions during sample collection.

### Immunofluorescent Imaging

Formalin fixed, paraffin wax embedded tissue was cut (7 μm) onto poly-L-lysine-coated slides, dried overnight at 55 °C, dewaxed in xylene and rehydrated through a graduated ethanol series (100%, 95%, 70%) and distilled water (dH<sub>2</sub>O). Antigen retrieval was performed by incubating the slides at 97 °C in 10 mM sodium citrate buffer (pH 6.0) for 30 minutes. The slides were left to cool to room temperature for 20 minutes before removal. Samples were rinsed briefly with PBS and non-specific binding was blocked using 1.5% fish skin gelatin (Sigma) in PBS containing 0.025% Triton-X-100 for 90 minutes at RT. Samples were incubated with primary antibodies (Keratin 1, 1:1000, Covance, Keratin-14, 1:1000, Covance, Loricrin, 1:500, Covance, Ki67, 1:1000 BD Biosciences) overnight at 4 °C. After appropriate washing with PBS, samples were incubated with a fluorophore-conjugated secondary antibody (Donkey anti-Rabbit 488, 1:500, Invitrogen) for 1 hour at RT. Slides were covered with cover-slips using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield) and samples were examined using a Zeiss LSM 5 Exciter confocal laser scanning microscope.

### Haematoxylin and Eosin Staining (H&E)

Following dewaxing (as described above) 7 μm sections were stained with Mayers Haematoxylin (Sigma) at RT for 3 minutes. Blue staining was achieved by rinsing in tap water while differentiation was achieved by rinsing in 1% acid ethanol. Counterstaining was achieved by rinsing with eosin (Sigma) for 30 seconds while dehydration was achieved by sequential washing with 95% ethanol, 100% ethanol and Histo-Clear (National Diagnostics). Slides were covered with cover-slips with DPX (Agar Scientific) and examined by light microscopy using a Zeiss Axioplan 2 microscope.

### Drug Treatment and Analyses of Transdermal Transport

The barrier function of the skin constructs was examined by adding 5 μl of FAM-tagged oligonucleotides solution on the topical surface of the skin constructs. After placing the devices on the rocking platform, 10 μl medium from both reservoirs was collected and replaced with 10 μl of fresh medium at specified

time points. The concentration of the dye in medium was calculated by quantifying total absorbance at 515 nm using fluorescence spectrometer.

In the drug toxicity study, doxorubicin (Sigma) at a clinically relevant concentration of 36 μM in culture medium was added into the reservoirs.<sup>12</sup> The doxorubicin containing medium was replaced every other day throughout the culture period. At specified time points, the samples were collected for the analysis of immunohistochemistry.

### Drug Transport Model and Estimation of Transport Parameters

We developed a transport model to estimate the transport properties of the HSEs. The model geometry and parameters are depicted in **Figure 1B**.  $J_{ss}$  is the steady state mass transfer rate of drug and can be defined as below according to Fick's law.

$$J_{ss} = K_p A_{skin} (C_{drug} - C_{bottom}) \quad (\text{Eqn. 1})$$

where

$$K_p = \frac{\phi_s D_s}{\delta_s} \quad (\text{Eqn. 2})$$

The overall drug permeability is a function of overall drug partition coefficient,  $\phi_s = C_{skin}/C_{media}$ , overall diffusion coefficient of drug in the skin ( $D_s$ ) and full skin thickness,  $\delta_s$ .  $A_{skin}$  is the surface area of the skin and  $C_{drug}$  and  $C_{bottom}$  are the drug concentrations in the vehicle and bottom chamber located underneath the membrane. Mass balance in the media and bottom chamber compartments yield:

$$\frac{dC_{bottom}}{dt} = \frac{Q_{media}(C_{media} - C_{bottom}) - J_{ss}}{V_{bottom}} \quad (\text{Eqn. 3})$$

$$\frac{dC_{media}}{dt} = \frac{Q_{media}(C_{bottom} - C_{media})}{V_{media}} \quad (\text{Eqn. 4})$$

$C_{media}$  is the concentration of the drug in the medium compartment.  $Q_{media}$  is the total volumetric flow rate, and  $V_{bottom}$  and  $V_{media}$  are the volume of bottom chamber and media. These two ordinary differential equations were solved simultaneously using ode45 function of Matlab v.2013 and by varying  $K_p$  to give the best fit to the experimental data. The initial ( $C_{Drug}$ ) and steady-state drug concentrations in the media were used to estimate the overall partition coefficient:

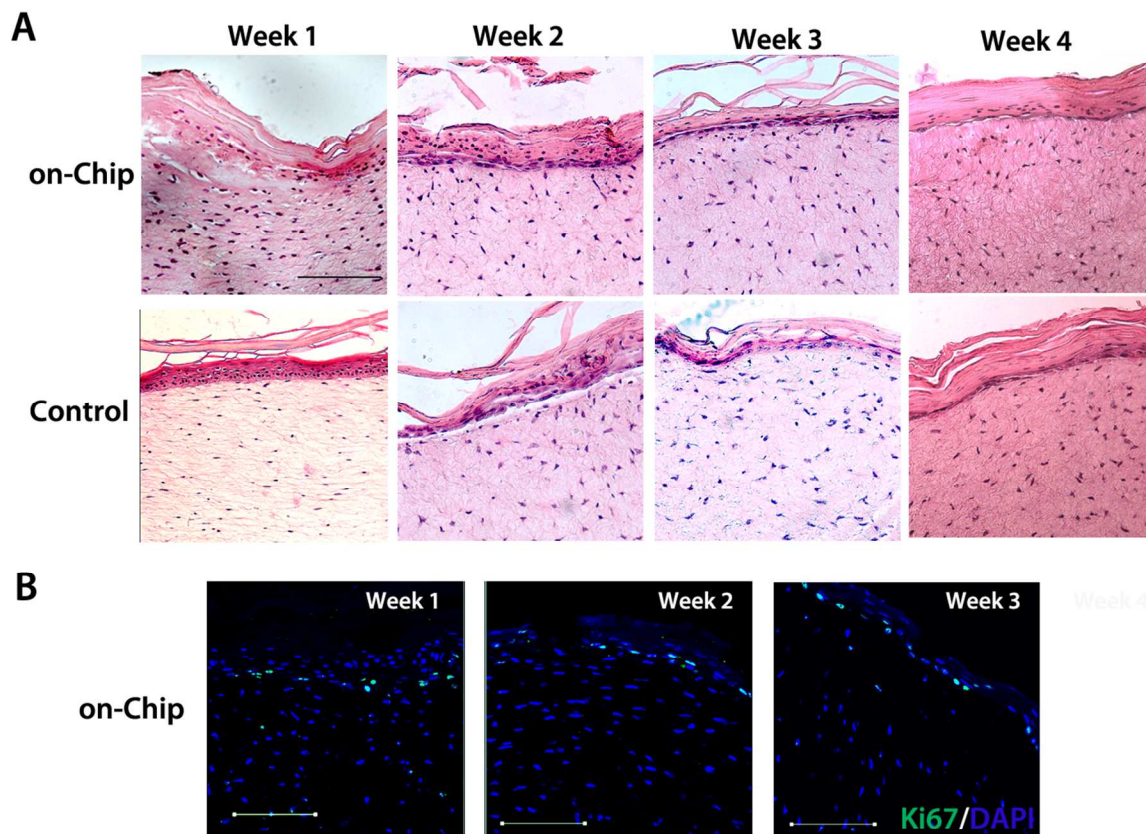
$$\phi_s = \frac{C_{Drug} V_{drug} - C_{media} V_{media}}{C_{media} V_{skin}} \quad (\text{Eqn. 5})$$

The drug diffusion coefficient was then calculated using the Eqn.2. To take the mass transfer resistance of the polycarbonate membrane into account, drug transport experiments were also run with the membrane alone without the HSEs.

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**Fig. 2 The duration of maintenance of HSEs-on-a-chip (A)** H&E staining of HSEs maintained HSE-on-a-chip or conventional cultures for 4 weeks, **(B)** Ki67 immunostaining of HSEs-on-a-chip to evaluate proliferation activity of basal keratinocytes. Scale bars: 100  $\mu\text{m}$

## Results and Discussion

The HSE-on-a-chip system was designed to allow pump-free, low-cost, and long term maintenance of HSEs for drug testing purposes. The dimensions of the microchannels were determined based on physiologically relevant residence times using previously reported blood perfusion rates and organ volumes for human adult skin.<sup>13</sup>

This approach of residence time based dimensioning was used to achieve physiological transport of molecules and to facilitate further integration of the HSE-on-a-chip with other organ-on-chip models, such as liver or GI-track. The gravity-driven flow rate of the culture medium was created and re-directed by placing the chip on a rocking platform as previously described.<sup>7</sup> Gravity-driven flow systems may cause unstable flow rates due to the change in hydrostatic pressure over time. To avoid this, we chose a cycling frequency of 2 cycles/min, which only causes a slight deviation of 0.3% in the flow rate (e.g.  $Q_{\text{avg}}=3.28 \mu\text{l}/\text{min}$ ) and

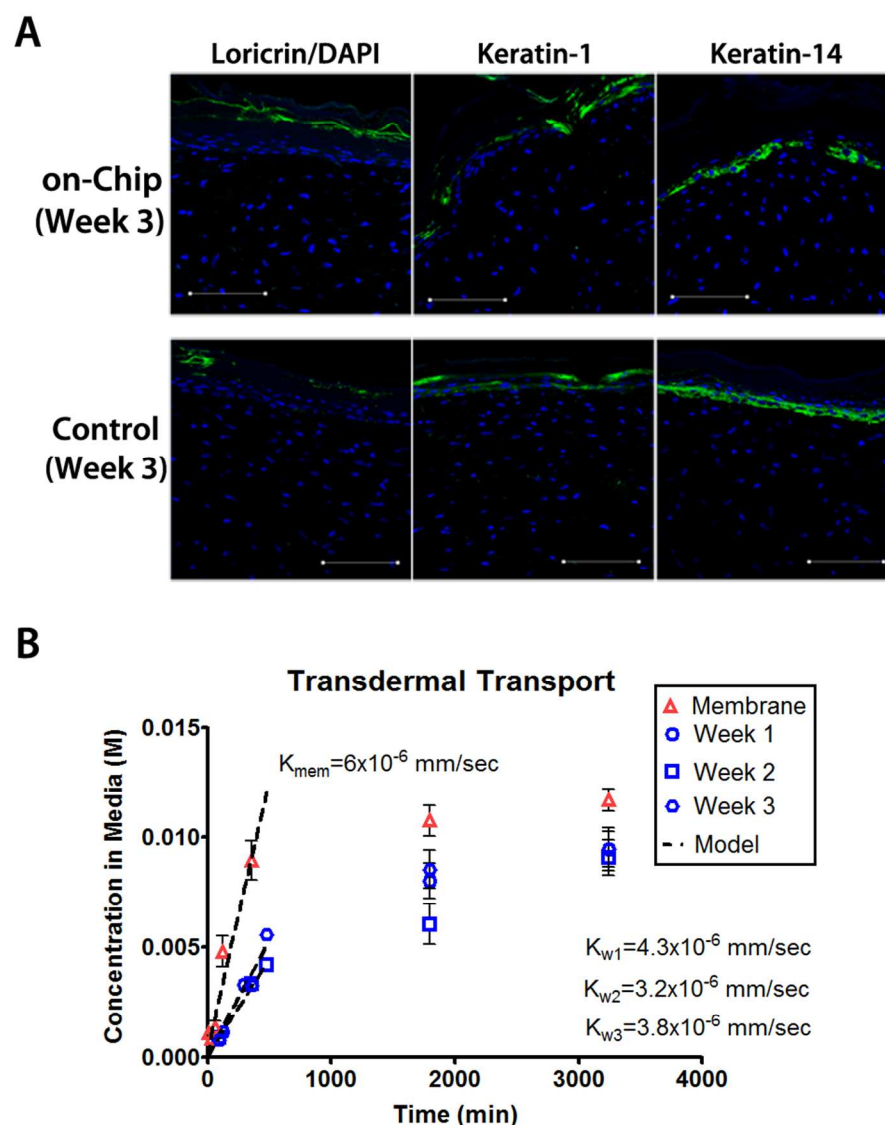
residence time ( $\tau_{\text{avg}}=8.5 \text{ min}$ ) over 30 second period of each cycle (**Supplementary Figure 1**). On the other hand, relatively lower cycling frequencies may cause non-uniform distribution of molecules due to inadequate time for the convective transfer of molecules across the channel length. In our system, the characteristic time for the convective transport along the channels was estimated to be 15 seconds. Therefore, a cycling time of 30 seconds allows for sufficient convective transfer to achieve a well-mixed system.

It is especially challenging to establish a stable air-epidermal interface in a gravity driven flow system. To address this, the HSE housing on the device was precisely leveled so that the top level of the medium in both reservoirs does not exceed the bottom layer of the HSE when the device is tilted on the rocking platform. The reservoir volume was also adjusted to accommodate sufficient medium for two days without a need for medium replacement. The HSE-on-a-chip operates with a minimal medium requirement of 125  $\mu\text{l}$  medium per day which is

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**Fig. 3 Long-term maintenance of HSEs-on-a-chip.** (A) Immunolabeled HSE-on-a-chip and control samples with layer specific markers, Keratin-1, Keratin-14 and Loricrin (green) and DAPI (blue) showing proper formation and structural integrity of epidermal layers after 3 weeks. Scale bars: 100  $\mu\text{m}$  (B) Transdermal transport of FAM-tagged oligonucleotides through HSE-on-a-chip in comparison to polycarbonate membrane. The overall permeability coefficients,  $K_p$ , of the HSEs were estimated using the transport model to evaluate the skin barrier function throughout 3 weeks.

nearly 36 times less amount of medium required in conventional transwell cultures, which facilitates measurement of chemicals passing the skin barrier.<sup>14</sup>

#### Long term maintenance of HSE-on-a-chip

We examined the longevity of the HSE-on-a-chip in comparison to the transwell plate control samples. The maintenance of the HSEs was assessed by the formation of desired dermal and epidermal layers, the integrity of each layer, as well as the proliferative activity of keratinocytes. The H&E staining of HSE

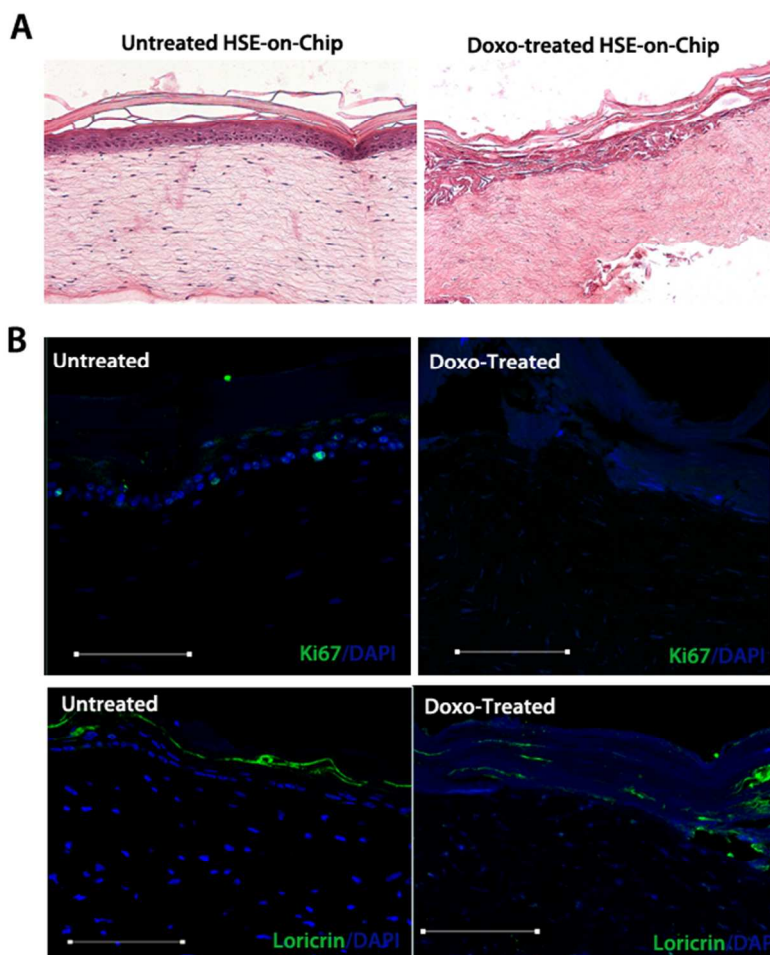
histology sections from Week 1 demonstrated the integrity of dermal and epidermal layers. The dermal-epidermal interface was formed after one week and conserved for 4 weeks on the chip and found to be comparable to the control (Figure 2A).

In healthy adult skin, keratinocytes located at the basal layer retain their proliferative activity. Therefore, to evaluate the proliferation of the basal keratinocytes, HSEs were stained for Ki67 over 4 week period. We detected the presence of proliferating keratinocytes up to 3 weeks both in HSE-on-a-chip and control samples (Figure 2B and Supplementary Figure 2A).

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**Fig. 4** The toxicity of doxorubicin ( $36\ \mu\text{M}$ ) on HSE-on-a-chip. **(A)** H&E staining of the HSE-on-a-chip after one week of doxorubicin treatment in comparison to untreated HSE-on-a-chip. **(B)** Immunostaining of Ki67 and Loricrin of HSE-on-a-chip treated with doxorubicin for a week. Scale bars:  $100\ \mu\text{m}$

5 After 4 weeks, however, we did not observe any Ki67 positive cells in neither the HSE-on-a-chip nor the control samples, suggesting that proliferative capacity of keratinocytes in the HSE model had been reached, and this limited capacity is not a result of the HSE-on-a-chip platform but rather a result of the HSE  
10 culture conditions. Therefore, we used week 3 samples as the latest time point for analyses.

To examine the proper formation of the epidermal layers, we immunofluorescently labeled the histological HSE sections by a stratum corneum-specific marker, Loricrin, a differentiation marker, Keratin-1, and a basal layer marker, Keratin-14, throughout 3 weeks. Loricrin-positive layer with the absence of nuclei exhibited the presence of a well-formed stratum corneum after 3 weeks in the device (**Figure 3A**). Similarly, Keratin-1 and Keratin-14 staining demonstrated that keratinocyte differentiation

20 and localization were established properly and comparable to the control throughout 3 weeks (**Figure 3A** and **Supplementary Figure 2B**). We further evaluated the progress in skin barrier function over 3 weeks by transdermal transport of FAM-tagged oligonucleotides. The measured drug concentration in the media  
25 over time showed that the permeability of oligonucleotides through the HSE after Week 1 is not significantly different than in Week 2 and 3. Using the mathematical model, we also estimated intrinsic properties, such as diffusion and partition coefficients, of the HSEs. The intrinsic properties remained  
30 similar throughout 3 weeks of maintenance on the chip (**Table 1**). These data demonstrated that skin barrier function can be maintained for 3 weeks on the chip. It should be noted that the selected rocking frequency is critical for transdermal drug transport and for estimating the transport parameters. For

example, low rocking frequencies can lead to unstable flow rates and affect the removal of the drug from the HSEs whereas high rocking frequencies can cause limited convectional transfer of the drug across the channel length and cause non-uniform distribution of the drug in the system. The rocking frequency of 2 cycles/min used in this study allows for a stable flow rate and a well-mixed system.

**Table 1** Estimated intrinsic transport parameters of the full-thickness HSE-on-a-chip for FAM-tagged oligonucleotides over three weeks

	Week 1	Week 2	Week 3
$K_p$ ( $\mu\text{m}/\text{sec}$ )	$4.3 \pm 0.9 \times 10^{-3}$	$3.2 \pm 0.6 \times 10^{-3}$	$3.8 \pm 0.8 \times 10^{-3}$
$\Theta_s$	$10.9 \pm 4.2$	$12.8 \pm 3.7$	$10.9 \pm 3.5$
$D_s$ ( $\mu\text{m}^2/\text{sec}$ )	$0.11 \pm 0.07$	$0.07 \pm 0.03$	$0.10 \pm 0.04$

### HSE-on-a-chip allows for drug testing

To validate the capability of the HSE-on-a-chip platform to be used in drug toxicity studies, we treated the HSE-on-a-chip with the anticancer drug, doxorubicin, at a clinically relevant concentration of  $36 \mu\text{M}$ , which is near the high end of the clinical range.<sup>12</sup> Doxorubicin is widely known to have toxic effects, including vacuolar necrosis of human keratinocytes, to human skin.<sup>15</sup> The results were compared with the untreated HSE-on-a-chip after one week of culture. The H&E and immunostaining of the samples showed that doxorubicin treatment causes a spatial detachment of the basal layer along the epidermal-dermal interface (**Figure 4A and Supplementary Figure 3**). In the untreated samples, the proliferative activity of the basal keratinocytes was preserved after one week, whereas the basal keratinocytes of the doxorubicin-treated HSEs exhibited no proliferation, supporting the previously shown anti-proliferative role of doxorubicin (**Figure 4B**).<sup>16</sup> We did not quantify the percentage of Ki67 positive cells for comparison since all cells in the doxorubicin-treated sample were Ki67 negative. Interestingly, the stratum corneum marker, Loricrin, was also observed in the basal and suprabasal layers of the doxorubicin treated HSEs, suggesting that doxorubicin treatment may result in premature epidermal differentiation of keratinocytes (**Figure 4B and Supplementary Figure 3**). Overall, this data demonstrates that high plasma concentrations of doxorubicin may have direct toxic effects on keratinocyte proliferation and differentiation. Thus, this effect should be considered as a potential complication limiting potentially the clinically used doses of doxorubicin.

### Conclusion

In this study, we demonstrated a microfluidic platform that allows for long term maintenance of HSEs in a micro-scale setting for the purpose of drug testing. The need for an external pump and tube connections makes conventional micro-scale drug-testing platforms difficult to operate, especially when large number of parallel drug screening experiments is required. Our HSE-on-a-chip platform has a unique capability compared to current skin-on-chip systems, specifically to re-circulate the medium at desired flow rates without a need for a pump. In addition, the HSE-on-a-chip, along with the mathematical model described

here, can be used to estimate intrinsic skin transport properties such as diffusion rate and drug partitioning, independent of skin thickness. This capability allows for making direct correlations to *in vivo* skin permeation data and predictions for human skin, since the species-specific variations observed in drug permeability studies usually depend on the discrepancies in skin composition and thickness. Although we only estimated overall transport properties for full-thickness skin, the design of the system is also suitable for operating at dermal or epidermal only settings to obtain layer-specific transport properties. Furthermore, HSE-on-a-chip platform was dimensioned based on physiologically relevant blood residence time in human skin, which makes it capable of integration into multiple-organs-on-chip systems and more comprehensive human-on-chip platforms. The HSE-on-a-chip is a powerful *in vitro* tool for skin drug testing studies and holds a great potential to be more predictive and patient-specific with further incorporation of other iPSC-derived skin cell type, such as melanocytes, as well as innervation by sensing neurons.

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### Notes and references

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